ROLE OF INFLAMMATION AND INFLAMMATORY MEDIATORS IN COLORECTAL CANCER

RAYMOND N. DUBOIS, MD, PHD

ABSTRACT

Chronic inflammation is a risk factor for several different cancers including colorectal cancer (CRC). However, the mechanisms underlying the contribution of inflammation to cancer remain elusive. Pro-inflammatory mediators such as cyclooxygenase 2 (COX-2) and prostaglandin E_2 (PGE_2) contribute to cancer progression. Here, we show that COX-2 is an immediate-early response gene induced by growth factors and pro-inflammatory cytokines and its levels are elevated in human CRCs. Furthermore, we show that COX-2−derived PGE_2 promotes colonic tumor growth via silencing certain tumor suppressors and DNA repair genes by DNA methylation in colonic epithelial tumor cells. We also report that C-X-C motif chemokine receptor 2 accelerates colonic inflammation and colitis-associated tumorigenesis by mediating myeloid-derived suppressor cell recruitment to the tumor microenvironment. These findings not only support a rationale to target these pro-inflammatory pathways for cancer prevention and treatment but also provide support for developing new therapeutic approaches to subvert chronic inflammation- and tumor-induced immunosuppression.

INTRODUCTION

Solid tumors, including the four most prevalent cancers worldwide (colon, breast, lung, and prostate cancers), are now considered as "abnormal" organoids that contain cancer cells as well as many other cell types. The tumor microenvironment consists of stromal cells, including carcinoma-associated fibroblasts, endothelial cells, and infiltrating immune cells. Cancer cells are able to switch a normal microenvironment to one that supports tumor growth and spread by inducing angiogenesis, inflammation, and immunosuppression.

It is widely accepted that cancer develops as a result of genetic and epigenetic alterations (1,2). In most cases, genetic mutations are acquired over the course a lifetime, but can also be inherited at birth. For

Correspondence and reprint requests: Raymond N. DuBois, MD, PhD, Executive Director of the Biodesign Institute at Arizona State University, PO Box 875001, 1001 S. McAllister Ave, Tempe, AZ 85287, Tel: 480-965-1228, Fax: 480-727-9550, E-mail: duboisrn@asu.edu.

example, colorectal cancer (CRC) is a heterogeneous disease, including at least three major forms: hereditary, sporadic, and colitis-associated CRC. Hereditary CRC accounts for approximately 10% to15% of CRC with at least two major types: familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Germ-line mutations in one allele of the adenomatous polyposis coli (*APC*) tumor suppressor gene are responsible for FAP. FAP patients have almost a 100% risk of developing CRC during their lifetime. The cause for HNPCC (also referred to as the Lynch syndrome) is due to inherited mutations in genes that encode DNA mismatch repair enzymes (primarily *MLH1* and *MSH2*). Patients with HNPCC have a 60% to 80% chance of developing cancer in their lifetime. Sporadic CRC accounts for more than 80% of CRC that occurs in the population and is dependent on a series of somatic mutations and/or epigenetic alterations that occur in different stages over time (3). For example, a somatic mutation of the *APC* gene occurs very early as the initiating event with high frequency (85%). Similarly, *KRAS* mutations usually arise at the stage of large adenomas with approximately 40% frequency, and mutations of *TP53* and *SMAD4* occur in the transition of large adenomas into invasive carcinomas of sporadic CRC. Moreover, epigenetic alterations represent another mechanism for silencing tumor suppressor and DNA repair genes. For example, hypermethylation of CpG islands located in tumor suppressor and DNA repair genes such as *P16*/*INK4a*, *P14*/*INK4b*, *APC*, *hMLH1,* and *MGMT* has been observed in sporadic CRC (4). Epidemiologic and experimental evidence strongly implicates chronic inflammation as a risk factor for developing CRC. Indeed, ulcerative colitis, a form of inflammatory bowel disease (IBD), is associated with an increased risk for developing CRC (5). More than 20% of patients with ulcerative colitis are reported to develop colitis-associated CRC (6).

CRC is the fourth most common malignant neoplasm and the second leading cause of cancer deaths in the United States. Although colonoscopy screening is an effective way to detect and prevent CRC by removing precancerous adenomas (7), 70% of CRC patients present to their physician with advanced disease, resulting in an unacceptable 5-year survival rate (8). Unfortunately, distant metastases are the major causes of death for patients with advanced CRC. Liver and lung metastases occur in approximately 20% to 70% and 10% to 20% of patients, respectively. The standard therapies for metastatic CRC have improved considerably but we continue to face an unacceptable 5-year survival rate. Clearly, prevention is the key to success for this disease. Epidemiologic evidence supports the rationale for CRC pre-

vention via changes of dietary patterns and exercise, as well as positive effects of administrating calcium supplementation (9). Moreover, chemoprevention has long been discussed as an approach for the population and individuals at high risk for developing cancer. Great effort has been expended to develop these drugs for both CRC prevention and treatment over the past 2 decades. Beneficial effects on reducing the risk of developing CRC have been found from using nonsteroidal anti-inflammatory drugs (NSAIDs), including a non-selective NSAID such as aspirin, and cyclooxygenase (COX)-2 selective inhibitors (COXIBs) such as celecoxib.

Epidemiologic and clinical studies have shown that long-term use of NSAIDs reduces the relative risk of CRC by 40% to 50% (10). Unlike COXIBs and other nonselective NSAIDs, long-term daily aspirin use is beneficial for prevention of both CRC and cardiovascular diseases. Daily use of aspirin significantly suppressed polyp growth in FAP patients (11) and substantially reduced cancer incidence in patients with Lynch syndrome (12). In sporadic CRC, four randomized controlled trials showed that aspirin use reduced risk of adenoma recurrence in patients with a history of colorectal adenomas (13–16). More intriguingly, recent observational and clinical studies revealed that daily use of aspirin was associated with a reduced risk of metastatic spread (17) and inhibited the spread of primary tumor cells to other organs after the diagnosis of localized disease, in particular CRC (18), suggesting the potential therapeutic efficacy of NSAIDs in advanced CRC. However, the molecular mechanisms underlying the effects of NSAIDs, including aspirin on reducing inflammation and protecting against cancer formation and progression, have remained elusive.

MATERIAL AND METHODS

Animal Models

All animal experiments conform to our animal protocols that were reviewed and approved by the Institutional Animal Care and Use Committee. *Cxcr2*$^{-/-}$ in BALBc genetic background and their littermate control (wild type [WT]) mice as well as *Apc*$^{Min/+}$ mice were obtained from the Jackson Laboratory. For prostaglandin E_2 (PGE_2) treatment, *Apc*^{min} mice at the age of 6 weeks were randomly placed into three groups treated with vehicle, 150 μg PGE_2/each mice, or 300 μg PGE_2/each mice by gavage feeding twice per day for 7 weeks. For azoxymethane/dextran sodium sulfate (AOM/DSS) treatment experiments, male mice for each genotype were given a single intraperitoneal

administration of AOM (10 mg/kg body weight). Seven days later, these mice were randomly divided into two groups fed with 1.25% DSS in drinking water for 4 cycles as shown in Figure 1A or water as control. At the end of the experiments, some of colons from each group were fixed for counting tumors and determining inflammation scores and the rest were used for examining profiles of immune cells. Histologic scoring of inflammation was determined as described previously (19).

Isolation of Immunocytes From Colonic Mucosa

For colonic immune cells, all the fat and Peyer's patches were removed from excised colon. Colonic tissues were cut into small pieces (2 to 3 mm). Intraepithelial immunocytes were separated by incubating tissues in 0.015% Dithioerythritol (Sigma-Aldrich, St Louis, MO). For isolating immunocytes from the lamina propria, the remaining tissue was incubated with ethylenediaminetetraacetic acid (EDTA). After incubation, EDTA was washed away, the tissue was minced and digested using a digestion buffer (RPMI medium containing 5% fetal bovine serum and 200 units/mL of collagenase) (Gibco, Grand Island, NY). A discontinuous (44% and 67%) Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) separation method was used to enrich immunocytes.

Flow Cytometry Analysis

Immunocytes isolated from the colon were incubated for 30 minutes on ice with the appropriate combination of the following antibodies in staining buffer (BioLegend, San Diego, CA) at the following dilution: CD45-APC (1:40), CD45-PB (1:200), or CD45-PECy7 (1:40), CD11b-FITC (1:11), CD3-PE or CD3-PerCP-Cy5.5 (1:40), CD8a-APC-Cy7 (1:40), CD4-PacBl or CD4-PerCp-Cy5.5 (1:40), Gr-1-Alexa700 (1:40), Ly6G-Alexa700 or Ly6G-PE (1:40), CD11c-PE-Cy7 (1:40), CD49b-APC (1:80), and F4/80-PE TexasRed (1:80), and CXCR2-PE or CXCR2-APC (1:4.5) as well as 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). Immune cell profiles were separated on a Gallios flow cytometer (Beckman Coulter, Brea, CA) as previously described (20). The flow cytometric profiles were analyzed using Kaluza software program (Beckman Coulter). DAPI or PI was used to exclude the dead cells during analysis of immune cell profiles.

Northern Blot Analysis

Rat intestinal epithelial (RIE) cells were grown in Dulbecco's Modified Eagle's (DME) supplemented with 10% fetal bovine solution, 2

mM L-glutamine, 100,000 U/L penicillin, and 100 mg/L streptomycin sulfate. Total RNA was isolated from RIE cells and human specimens using a standard guanidinium thiocyanate/acid phenol/chloroform/isoamyl alcohol extraction method with subsequent poly(A)$^+$ RNA selection by oligodeoxythymidylate chromatography. RNA samples were electrophoresed in denaturing agarose gels and transferred to nitrocellulose. Nitrocellulose blots were hybridized with ^{32}P-labeled cDNAs encoding prostaglandin H synthase-2 (COX-2).

Statistical Analysis

Each in vitro experiment was performed at least three times and each in vivo experiment was conducted at least twice. Comparisons among multiple groups were performed by factorial analysis of variance, followed by the Bonferroni test. Comparisons between two groups were performed with the Student *t*-test or Mann-Whitney U test where appropriate. Fisher's exact test was used for categorical variables and $P < .05$ was considered significant.

RESULTS

COX-2 Expression is Induced by Pro-inflammatory Cytokines and Elevated in Human CRC and Polyps

Although several hypotheses have been proposed in an attempt to explain the effects of NSAIDs, including aspirin and COXIBs, on reducing cancer risk and mortality, the most compelling evidence suggests that the effects of these agents are due to reduction of PGE_2 production by targeting activity of COX enzymes including COX-1 and/or COX-2. COX enzymes convert arachidonic acid into an endoperoxide intermediate that can be further metabolized to five structurally related prostanoids, including prostaglandins (PGs) such as PGE_2, PGD_2, $PGF_2\alpha$, PGI_2, and thromboxane A_2 via specific PG synthases. COX-1 is constitutively expressed in most tissues and was considered to represent a "housekeeping" enzyme responsible for maintaining basal prostanoid levels important for tissue homeostasis and platelet function. Our laboratory was the first to report that COX-2 expression is significantly induced by a growth factor, TGFα, in REI cells and dramatically elevated in CRCs and adenomas (Figure 1). These results show that COX-2 is an immediate-early response gene that is normally absent from normal intestinal cells and tissues but is highly induced by pro-inflammatory cytokines and is elevated in tu-

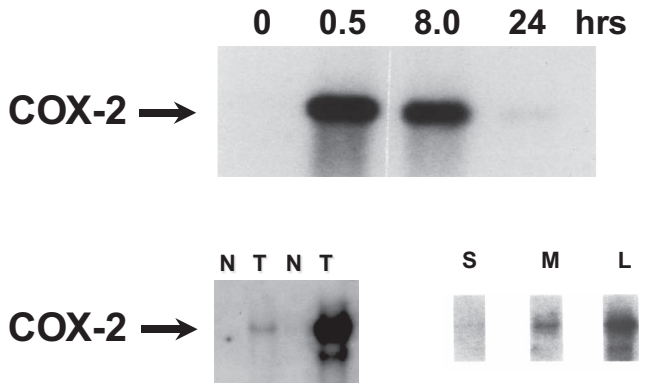

FIG. 1. COX-2 mRNA levels in RIE cells and human colorectal carcinomas and adenomas. Northern blotting was performed to analyze COX-2 mRNA isolated from RIE-l cells treated with TGFα (10 ng/mL) at the indicated times and from paired normal mucosa and carcinomas as well as adenomas (Abbreviations: N, normal mucosa; T, carcinoma; S, small adenoma; M, middle adenoma; and L, large adenoma).

mors. These findings were published in the *Journal of Clinical Investigation* and *Gastroenterology* (21,22). Multiple follow-up studies revealed that COX-2 expression is elevated in up to 90% of CRCs and 50% of adenomas (23) and its expression is associated with a lower survival rate among CRC patients (24).

COX-2-derived PGE_2 Promotes Intestinal Adenoma Growth

The biological effects of COX-2 depend on which type COX-2–derived prostanoids are produced in cancers. PGE_2 is the most abundant prostaglandin found in various types of human malignancies including colorectal, lung, breast, head, and neck cancers and its presence is often associated with a poor prognosis (25–28). However, previously there was no direct evidence showing that PGE_2 promotes CRC progression. We presented the first evidence showing that treatment of Apc^{Min} mice with PGE_2 resulted in promotion of colonic adenoma growth (Figure 2). The $Apc^{Min/+}$ mouse carries a point mutation in one *Apc* allele and spontaneously develops intestinal adenomas. It is a frequently used model for human FAP and sporadic CRC. Our result showing that PGE_2 accelerates colonic tumor growth has been published in *Cancer Cell* (29). Furthermore, our work further revealed that PGE_2 silenced certain tumor suppressors and DNA repair genes through DNA methylation to accelerate tumor formation and growth in $Apc^{Min/+}$ mice (30) (data not shown). This finding uncovers a previously unrecognized role of PGE_2 in the acceleration of tumor growth.

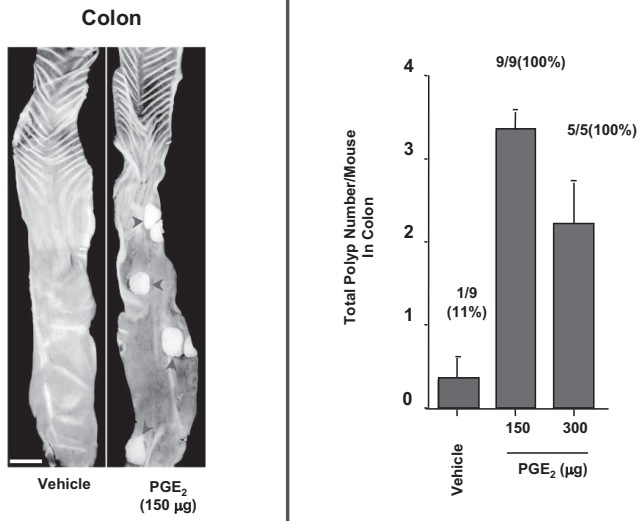

FIG. 2. PGE_2 accelerates colonic polyp growth. Gross view of colonic polyps in Apc^{min} mice treated with vehicle or PGE_2 (scale bar, 0.5 cm). Polyps from mice treated with vehicle or the indicated dose of PGE_2 were counted using a dissecting microscope. Data are expressed as mean ± SE.

Loss of CXCR2 Attenuates DSS-induced Chronic Inflammation and AOM/DSS-induced Tumor Burden

Our group previously identified CXCL1 as a novel PGE_2 downstream target and found that its expression correlated with PGE_2 levels in human CRC (31). CXCL1 belongs to the ELR^+ CXC chemokine family that is crucial for the recruitment of neutrophils to sites of inflammation. CXCL1 exerts its biological effects by binding to its cognate cell surface receptor (CXCR2) that belongs to the G protein-coupled receptor family. Moreover, we have shown that CXCR2 is elevated mainly in endothelial and immune cells found in human CRCs (31). These results indicate that PGE_2 might influence the biological function of stromal compartments, including immune cells, endothelial cells, and other cells via the CXCL1-CXCR2 axis. To examine whether the PGE_2-CXCL1-CXCR2 pathway is involved in chronic inflammation and colitis-associated tumorigenesis, WT or $Cxcr2^{-/-}$ mice were treated with AOM and DSS as outlined in Figure 3A. Repeated administration of DSS induced chronic inflammation in WT mice but not in $Cxcr2^{-/-}$ mice (Figures 3B-C). In addition, loss of *Cxcr2* dramatically reduced

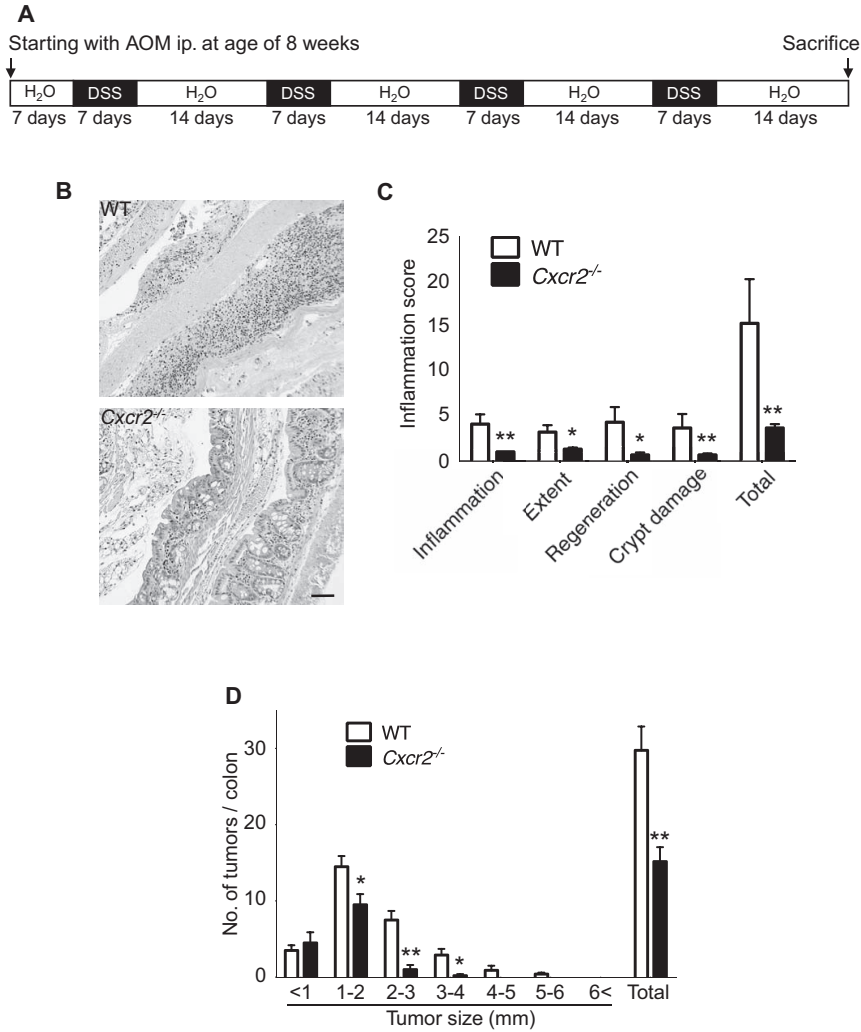

FIG. 3. Deletion of *Cxcr2* attenuates AOM/DSS-induced colonic chronic inflammation and colitis-associated tumor formation and growth. **(A)** Schematic of mice treated with AOM and DSS. **(B)** Representative of hematoxylin and eosin−stained sections from WT (*top panel*) and *Cxcr2* null mice (*bottom panel)* treated with AOM/DSS (scale bar = 100 μm). **(C)** Blinded histologic scoring of inflammation in colonic mucosa of mice was performed as described in Materials and Methods. **(E)** Tumor number and size were measured under a dissecting microscope. Data are represented as mean ± SEM (8 mice for each group). Asterisks represent statistical differences (*$P < .05$, **$P < .01$).

the tumor burden in these mice (Figure 3D). The severity of chronic inflammation directly correlated with tumor multiplicity (data not shown). These results show that CXCR2 is required to promote chronic inflammation and tumor burden in the large intestine and have been recently published in *Cancer Cell* (32).

Deletion of *Cxcr2* Diminishes DSS-induced Massive Infiltration of Myeloid-derived Suppressor Cells into Colonic Inflamed Mucosa

To examine whether CXCR2 is involved in recruitment of neutrophils and other immune cell, we first analyzed immune cell profiles in the colonic mucosa of mice treated with DSS for 4 cycles. Deletion of *Cxcr2* did not affect repeated DSS-induced infiltration of dendritic cells, T cells, natural killer (NK) NK cells, and NK T cells into the colonic mucosa but resulted in a trend toward reduction of infiltration of macrophages and neutrophils during the chronic phase (data not shown), although alteration of CXCR2 clearly affected infiltration of neutrophils in the acute phase (data not shown). In contrast, loss of CXCR2 dramatically suppressed a massive infiltration of myeloid-derived suppressor cells (MDSCs) ($CD11b^{+}Ly6G^{high}CD11c^{-}F4/80^{-}$) into the colon in both normal (water as control) and chronic inflammatory (DSS treatment) conditions (Figure 4). These results show that CXCR2 is required for homing of MDSCs from the circulatory system to inflamed colonic mucosa and have been recently published in *Cancer Cell* (32).

DISCUSSION

Epidemiologic studies revealed that regular use of aspirin reduces the risk of a subgroup of patients whose colon tumors expressed COX-2 at higher levels (33) and its use after the diagnosis of CRC at stages I, II, and III prolonged overall survival, especially among individuals whose tumors overexpress COX-2 (34). These findings suggest that the preventive and inhibitory effects of aspirin on CRC might depend on the presence of COX-2. Direct evidence supporting this hypothesis comes from studies showing that deletion of the COX-2 gene results in decreased tumor formation in both the small intestine and colon of Apc^{Min} mice (35). Similarly, COX-2 is induced in the large intestinal epithelium in active human IBD and in inflamed tissues of interleukin-10−deficient mice (a mouse model of IBD) (36,37). Therefore, metabolism of arachidonic acid by COX-2 may serve as one mechanism for the contribution of chronic inflammation to carcinogenesis (38).

Since measurement of a urinary PGE_2 metabolite (PGE-M) is an

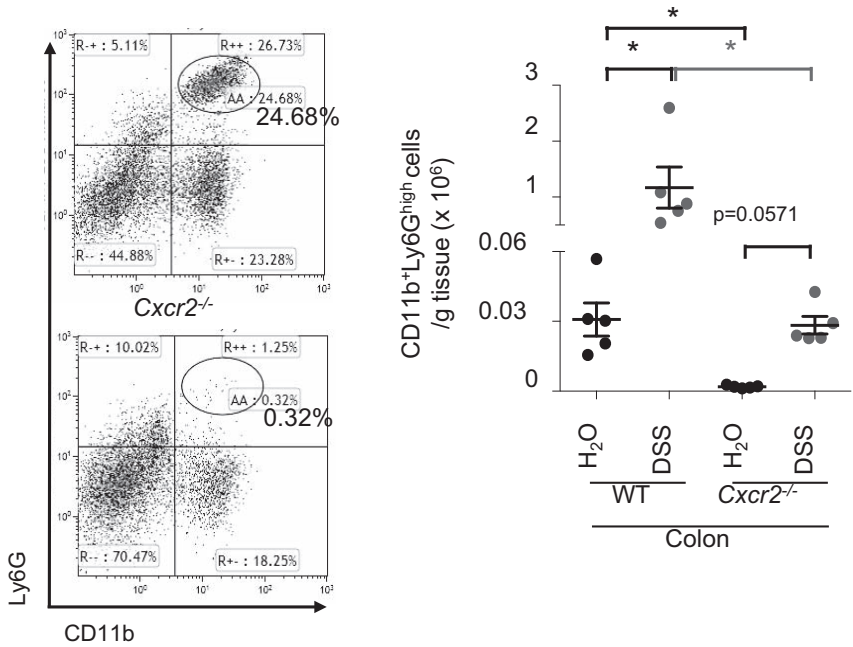

FIG. 4. Loss of CXCR2 inhibits DSS-induced massive infiltration of MDSCs into the colon. The indicated genotypic mice aged 8 weeks were treated with 4 cycles of 1.25% DSS and the cells isolated from colon were subjected to flow cytometry analysis. Viable granulocytes/monocytes or total cells were gated in a FSC/SSC plot. The subpopulation of MDSCs in colonic mucosa was represented as percentage of gated granulocytes/monocytes cells (*left panel*) or as the numbers of MDSCs per gram of each mouse colon tissue (*right panel*). Each dot in the right panel represents the numbers of MDSCs in colonic mucosa taken from one mouse.

effective way to quantify systemic PGE_2 production in vivo, efforts have been made to evaluate whether urinary PGE-M levels could serve as a promising biomarker for predicting cancer risk and prognosis, including CRC. Emerging epidemiologic evidence and a phase II biomarker study showed that urinary PGE-M levels were associated with an increased risk of developing colorectal (39–41), gastric (42), and breast cancer (43), as well as breast cancer metastasis (44) and disease progression or death in patients diagnosed with head and neck squamous cell carcinomas (44,45). Our results provide the first direct evidence showing that PGE_2 promotes colonic tumor growth in Apc^{Min} mice (Figure 2). Multiple follow-up studies performed by other investigators revealed that elevated endogenous PGE_2 via genetic deletion of *15-Pgdh* promoted colon tumor growth in $Apc^{Min/+}$ and AOM mouse models (46). In contrast, inhibition of endogenous PGE_2 via genetic

deletion of prostaglandin E synthase (*Ptges*) suppresses intestinal tumorigenesis in $Apc^{Min/+}$ and AOM models (47). These results further support a hypothesis that COX-2–derived PGE_2 contributes to human CRC formation and progression. More importantly, epidemiologic results showed that levels of urinary PGE-M in healthy humans (48) and breast cancer patients (43,44) were suppressed significantly not only by treatment with nonselective NSAIDs, including aspirin, but also by COXIBs, suggesting that the majority of PGE_2 formed in vivo may be derived from COX-2. Phase II studies also revealed that non–small cell lung cancer (NSCLC) patients with complete and partial responses to adjuvant therapy with paclitaxel, carboplatin, and celecoxib experienced a significant decrease in the level of urinary PGE-M (49) and recurrent NSCLC patients with the greatest proportional decline in urinary PGE-M levels experienced a longer survival compared to those with no change or an increase in PGE-M when treated with celecoxib and docetaxel (50). Taken together, these findings suggest that the anti-tumor effects of NSAIDs depend on reduction of PGE_2 production via targeting COX-2.

The levels of CXCR2 ligands correlate with the inflammatory state in IBD patients and are elevated in sporadic CRC in humans (51), indicating that the CXCR2 ligands and the receptor, CXCR2, may play a role in IBD and CRC. Our studies reveal for the first time that CXCR2 promotes colonic chronic inflammation and colitis-associated tumorigenesis in vivo (Figure 3) and is required for recruitment of MDSCs into colonic mucosa (Figure 4). Moreover, our recent results show that PGE_2 induces CXCR2 ligand expression in intestinal mucosa and tumors (32). These results indicate that PGE_2 may promote homing of CXCR2-expressing MDSCs into the colon via induction of CXCR2 ligands in colon mucosa. Further studies are required to test our hypothesis.

MDSCs represent a heterogenous population of immature myeloid cells. The levels of MDSCs in the blood are positively correlated with clinical cancer stage and metastatic tumor burden in mice and patients including colon cancers (52,53). MDSCs have been shown to contribute to cancer immune evasion via suppressing T cell activation, proliferation, trafficking, and viability, inhibiting NK cells, and promoting activation and expansion of Foxp3-positive Treg cells (54). Our recent study showed that colonic MDSCs recruited by chronic inflammation promote colitis-associated tumor formation, growth, and progression via suppression of colonic $CD8^+$ T cell cytotoxicity against tumor cells in a mouse model of IBD-associated carcinomas (32). Collectively, our findings uncover a previously unrecognized role of CXCR2 in recruit-

ing MDSCs into inflamed colonic mucosa and colitis-associated tumor from the circulatory system, but also reveal a novel function of MDSCs in connecting chronic inflammation to cancer.

In summary, COX-2 is an immediate-early response gene induced by pro-inflammatory cytokines and is elevated in human CRC. COX-2–derived PGE_2 promotes colonic tumor growth by affecting DNA methylation. Our recent findings reveal how MDSCs are recruited to local inflamed tissues and the tumor microenvironment and the mechanism by which local MDSCs contribute to CRC progression. Moreover, our results showing PGE_2 induction of CXCR2 ligands in colonic mucosa and tumors suggest that PGE_2 might promote tumor formation and progression by recruiting CXCR2-expressing MDSCs via induction of CXCR2 ligands. Our work not only sheds light on how the inflammatory microenvironment contributes to cancer immune evasion by allowing tumor cells to escape from immunosurveillance, but also provide a rationale for developing therapeutic approaches to subvert chronic inflammation- and tumor-induced immunosuppression by using CXCR2 antagonists and/or neutralizing antibodies.

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DISCUSSION

Quesenberry, Providence: Great talk. Some of the evolving thinking, I think, in cancer and especially colorectal cancer puts mutations as important but perhaps not primary. What was particularly interesting, I think, was some recent data looking at cytokeratin-positive cells in marrow before overt metastasis — the presumed metastatic cells, which is what kills patients — and finding that they have minimal numbers of mutations compared to the primary tumor. I think your work fits in this really nicely that the objective probably should be to go after these very early metastatic cells, which are not represented very well by the evolution of the primary tumor. I'd like your comments.

DuBois, Tempe: I think that I agree with that concept. I think that the other thing that we are finding is that some of the mutations that confer resistance to targeted treatments are already present even before that treatment is given. So some of these are already predetermined and clearly there are compartments within the body, you know, where some of these cells reside that are quite different. It's very heterogeneous. It's not just one clonal type of cell like was generally thought.

Quesenberry, Providence: I agree with you totally. A major part of what we may be dealing with is not the primary tumor cell but the environment. The tissue organization field theory is one that relates to that.

DuBois, Tempe: I think one of the things we've definitely seen is this tumor microenvironment with these inflammatory mediators. The microbiome also has an effect. I didn't have time to talk about that today. Clearly that elaborates molecules that stimulate the immune system and inflammation, and this creates a niche within this tumor microenvironment that is completely separate from the mutations.

Oates, Vanderbilt: That was a great talk. Apropos the metastasis issue, I think it's striking in the aspirin trials that were reported by Rothwell and colleagues — the metastasis of tumors that developed during aspirin trials compared to placebo — colon cancer metastasis dropped by more than 80%. It's the most striking effect on metastasis that you can imagine. It drops to a level that is about to the level of noncompliance of aspirin therapy, so it's approaching 100% if people you really estimate would be taking their aspirin. Thinking about the effect of aspirin on metastasis, there is a microenvironment for the metastatic cell as well as the primary tumor. That microenvironment includes intense involvement of the platelet, which surrounds the tumor cells and facilitates it's metastatic spread including work that we've recently done that shows that when platelets interact with these metastatic cells that it actually turns on PGE_2 productions to elicit some of the effects you described.

DuBois, Tempe: I agree Dr Oates. I think that the data is pretty remarkable when you take individuals who have undergone treatment for colorectal cancer and treat a group with aspirin. Their recurrence rate is much decreased, and I think there are a lot of issues going on there. We are trying to discern what those are through these animal models, but ultimately probably human studies are going to be needed to really understand that.

Berger, Cleveland: It's interesting to see how we have come now almost full circle and we are considering the inflammatory component of cancer again. Would you comment on the use of aspirin or other anti-inflammatory agents as part of therapy for cancer? You talked about its effect as a preventive agent. What about therapeutic?

DuBois, Tempe: Well there are a couple of CALGB (Cancer and Leukemia Group B) studies that are underway looking at the use of some of these agents in a neoadjuvant-type setting. So they're going to be a couple more years before those have accrued and are done. But I think people are exploring that type of approach to see if it could have some positive effect on the therapeutics there. The idea of using aspirin or NSAIDS for the population, obviously there are side effects of these drugs. They cause GI toxicity, bleeding, and the selective inhibitors can cause cardiovascular side effects. So I think we have to be very careful there, and it all depends on the relative risk of the disease versus the benefit of taking these drugs.

Pasche, Birmingham: Very nice talk, Ray. Just to follow on the previous question. There is now solid data that aspirin given after successful resection of a stage III colon cancer, for example, actually significantly decreases the risk of progression and metastasis. There was one study published in New England by the Ogino group about a year and a half ago (55), and I actually wrote the editorial for the *New England Journal* (56) on that, and one that just was published in the *Journal of Clinical Oncology* last week that confirmed this finding (57). So I think we now have solid data to offer aspirin to patients after successful surgery in those who have mutation on the PIK3CA gene. The *JCO* paper reported which sequencing method they did. It's fairly easy. It's easy to offer to patients. I think, with the relatively benign interventions such as aspirin treatment after surgery, we have, at least today, enough evidence to me. At least that's what I do for my patients with recently diagnosed colon cancer.